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Atty Docket No: 407E-914400US
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Donna G. Albertson; Daniel Pinkel; Antoine Snijders
Application No.: 09/574,386

Filed: 5/19/2000

For: **Methods And Compositions For Preparation Of A Polynucleotide Array**

Examiner: Spiegler, A.

Art Unit: 1656

DECLARATION UNDER 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

DECLARATION OF DR. DONNA G. ALBERTSON

I, Dr. Donna G. Albertson, am an Associate Professor in Residence in the Cancer Research Institute and the Department of Laboratory Medicine at the University of California, San Francisco, and a co-inventor of the above-referenced application. This Declaration provides a figure showing an agarose gel analysis of ligation-mediated PCR of BAC DNA, which was carried out essentially as described in Example 1 of the above-referenced application. Specifically, ligation-mediated PCR was carried out according to the following protocol, which was used to prepare 96 DNA spotting solutions from pure BAC DNA:

Restriction Enzyme Digest of BAC DNA

The restriction enzyme digest is carried out in a 5 μl reaction volume containing: 2.2x One-Phor-All Buffer Plus™, 2 U Mse-I restriction enzyme and 20-500 ng BAC DNA. The reaction is set up as follows:

1. Dilute the 10x One-Phor-All Buffer Plus™ to a final concentration of 0.8x in a volume of 750 μL using sterile H_2O and dispense 93 μl into each tube of an 8-tube strip.

2. Using a multichannel pipettor, dispense 2.5 μL of the 0.8x buffer solution into each tube of twelve 8-tube PCR strips and seal the tubes using a 96-well plate seal to prevent vaporation and possible contamination.

3. Add 1.5 μL BAC DNA to each tube using a single channel pipettor. The BAC DNA concentration usually ranges from 20-400 ng/μL. During this step only one 8-tube PCR strip is handled at a time. The remaining eleven 8-tube strips remain covered using a 96-well plate seal. After adding the BAC DNA to each 8-tube strip, place the strip in a different rack and seal with a 96-well plate seal.

4. Dilute the MseI restriction enzyme to a final concentration of 2 U/μL in a volume of 120 μL using 10x One-Phor-All Buffer Plus™. Keep the enzyme and the dilution on ice during this process.

5. Dispense 1 μL (2 U) of the MseI enzyme dilution into each tube individually; the enzyme should stay on ice during this process. Cap each 8-tube strip using an 8-cap strip after adding the enzyme and place on ice.

6. The reaction is placed in a PCR machine for an overnight incubation at 37°C (12-16 hours).

7. 1.75 μL of the digests can be run on a conventional 1% agarose gel containing 0.5 μg/mL ethidium bromide along with a φX174 RF DNA/Hae III Marker to check fragment length.

Ligation of Specific Primers to BAC DNA

The ligation reaction is carried out in a 10 μL reaction volume containing: 5 μM Primer 1, 5 μM Primer 2, 0.5x One-Phor-All Buffer Plus™, 1 mM ATP, 5 U T4 DNA ligase and 1 ng digested BAC DNA.

1. 1 μL of each of the restriction digests from the preceding step is diluted to 1 ng/μL using sterile H_2O. Determine the DNA concentration in each digest. Calculate the amount of sterile H_2O required to dilute 1 μL of each digest to a final DNA concentration of 1 ng/μL (for example, if the BAC DNA concentration in the digest reaction is X ng/μL, the appropriate amount of sterile H_2O to add is (X-1) μL). Set up twelve new 8-tube strips: individually add the calculated amount of sterile H_2O to each new tube at the locations corresponding to the locations of each digest. Transfer 1 μL of each digest using a multichannel pipettor. Cover using a 96-well plate seal.

2. The ligation reaction is set-up in a 1.5 mL tube by combining:

a. 56 μL Primer 1 (100 μM)

b. 56 μL Primer 2 (100 μM)

c. 56 μL One-Phor-All Buffer Plus™

d. 616 μL sterile H_2O

784 μL total

Mix by pipetting and dispense 98 μL into each tube of an 8-tube strip.

3. Using a multichannel pipettor, dispense 7 μL of the above prepared primer solution onto the bottom of twelve new 8-tube strips. Seal the 8-tube strips using a 96-well plate seal.

4. Add 1 μL of the 1 ng/μL BAC DNA digest prepared above to the 7 μL primer solution in each corresponding 8-tube strip.

5. Put the twelve 8-tube strips into a PCR machine. The annealing reaction is carried out at 65°C for 1 min, then the temperature is shifted down to 15°C, with a ramp of ~1.3°C/min (in a Perkin-Elmer 9700 PCR machine this is a ramp rate of 5%).

6. During the annealing process, dispense ~62.5 μL of 10 mM ATP solution into each tube of an 8-tube strip. Cap the 8-tube strip and place on ice.

7. As soon as the PCR machine reaches 15°C, promptly take out the tubes from the PCR machine and carefully open all 8-tube strips, including the 8-tube strip containing the ATP solution. Using a multichannel pipettor on the repeat-pipetting-mode, pick up 12.5 μL of 10 mM ATP and dispense 1 μL on the inside wall of each of the 96 tubes containing DNA. Gently tap the PCR rack so that the ATP slides into the DNA/primer solution. Seal with a 96-well plate seal. This procedure for adding the ATP reduces the probability of carry over contamination.

8. Dispense 1 μL (5 U) of the T4 DNA ligase enzyme into each tube of the 96 tubes individually. Cap each 8-tube strip using an 8-cap strip after adding the enzyme and place on ice.

9. The reaction is placed in a PCR machine for an overnight incubation at 15°C (12-16 hours).

Ligation Mediated PCR

The ligation mediated PCR is carried out in a reaction volume of 50 μL containing: 0.6x 10xPCR buffer #1, 0.4 mM dNTP mixture, 3.5 U DNA polymerase mixture and 10 μL ligation mixture .

1. Combine in a 15 mL tube:
   a. 336 μL 10xPCR buffer #1
   b. 224 μL dNTP mixture (10 mM)
   c. 3920 μL sterile H_2O

   4480 μL total

   Mix briefly by vortexing and place on ice.

2. Remove the ligations prepared above from the PCR machine. Pour the diluted dNTP mixture into a reservoir. Open all 8-tube strips carefully. Using a multichannel pipettor on the repeat-pipetting-mode, pick up 205 μL of the diluted dNTP mixture and dispense 40 μL on the inside wall of five subsequent 8-tube PCR strips. Purge the remaining 5 μL PCR mixture back into the reservoir. Discard the pipet tips. Repeat this procedure for the next five subsequent 8-tube PCR strips. Again, purge the remaining 5 μL diluted dNTP mixture back into the reservoir. Adjust the multi-channel pipettor fill volume to 85 μL, leaving the dispense volume at 40 μL. Pick up 85 μL diluted dNTP mixture from the reservoir and dispense 40 μL on the inside wall of the remaining two 8-tube PCR strips. Gently tap the PCR rack so that the diluted dNTP mixture slides into the DNA/primer solution. Seal with 8-tube strips.

3. To melt off Primer 2, place PCR tubes in the PCR machine at 68°C for 4 minutes. After 4 minutes, take out the tubes and add 1 μL of DNA polymerase mixture (3.5 U/μL) to each tube individually. Open one 8-tube strip at a time and close immediately after adding the enzyme mix and place on ice.

4. Place the 8-tube strips in the PCR machine: 68°C for 3 minutes; 94°C for 40 sec, 57°C for 30 sec, 68°C for 1 minute 15 sec for 14 cycles, 94°C for 40 sec, 57°C for 30 sec, 68°C for 1 minute 45 sec for 34 cycles and 94 °C for 40 sec, 57°C for 30 sec and 68°C for 5 minutes for the last cycle, followed by incubation at 4°C.

5. 3.5 μL of each PCR should be run on a conventional 1% agarose gel containing 0.5 μg/mL ethidium bromide along with a φ X174 RF DNA/Hae III Marker to check fragment length.

Figure

In the attached figure, panel 1 shows the typical size range and DNA distributions of three different MseI digest reactions, and panel 2 shows three ligation mediated PCR reactions. All DNA samples were separated on a 1% agarose gel containing 0.5 μg/mL ethidium bromide together with a φ X174 RF DNA/Hae III DNA Marker, which has a size range from 72 to 1353 bp. Panel 2 demonstrates that ligation-mediated PCR performed as described in the application produces a "smear" of DNA ranging in size from about 70 to 1500 bp. This is distinguished from the method described by *Smith et al.* (PCR Methods and Applications (1992) 2:21-27), in which individual fragments were amplified. See, e.g., Smith's Figs. 2-4, in which the amplification products appear as discrete bands.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's signature:

Donna G. Albertson

Donna G. Albertson, Ph.D.

Aug. 1, 2002

Date